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LIPID BIOSYNTHESIS BY CHLOROPLASTS ISOLATED FROM DEVELOPING *ZEA MAYS*

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Key Word Index *Zea mays*; Gramineae; maize; differentiating chloroplasts; acetate incorporation; fatty acid biosynthesis; stimulation by non-chloroplastic fractions.

Abstract—Fatty acid biosynthesis by isolated plastids has been examined in relation to chloroplast development and differentiation in leaves of maize plants grown in light for 7 days. Biosynthesis of fatty acids from acetate by proplastids prepared from the basal regions of the leaf was low and mainly palmitate was synthesized. The greatly increased utilization of acetate for fatty acid biosynthesis as the plastids increased in size was due to an increased synthesis of oleate. The maximum synthesis of total fatty acids and monoenoic fatty acids was obtained in chloroplasts prepared from leaf tissue 6–8 cm from the base of the plant where granal formation was most active. Fully-developed chloroplasts prepared from distal regions of the leaf were less active in fatty acid biosynthesis. Maize chloroplasts failed to synthesize fatty acids when isolated by methods commonly used to prepare active spinach chloroplasts. The method of isolation which included a density gradient gave a high proportion of Class I chloroplasts from maize leaves and incorporated up to about 10% of the acetate used. Biosynthesis of unsaturated fatty acids, especially with chloroplasts prepared from the most mature tissue, was increased by the addition of both mitochondrial and microsomal fractions. Increases in polyunsaturated fatty acids were also obtained but the proportions in the newly-synthesized fatty acids were well below the endogenous levels. Monoenoic synthesis was greatly stimulated by increasing the pH in the range 7·0–8·0 and also the highest proportions of unsaturated fatty acids were obtained at short incubation times.

INTRODUCTION

INTEREST in the capacity of chloroplasts for fatty acid synthesis during differentiation and granal formation arises from the considerable increase in their lipid content which occurs at this phase of development.[1,2] An extensive study of this relationship has been carried out by Stumpf *et al.* using chloroplasts isolated from dark-grown barley seedlings which had been greened for various lengths of time.[3] However proplastid development in a normal green leaf differs from the etiolated system in that in the former there is no extensive formation of prolamellar bodies.[4] Successive sections from the base to the tip of normal light-grown monocotyledonous leaves represent successive steps in cell and plastid differentiation and 7-day-old maize seedlings provide convenient tissue to study morphological and biochemical changes associated with plastid differentiation.[2] Subsequent examination of the fatty acid composition and the ability of the tissue from these sections of maize leaves to utilize acetate for fatty acid and lipid synthesis showed a marked intersectional

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[1] APPLEQVIST, L. A., BOYNTON, J. E., STUMPF, P. K. and VON WETTSTEIN, D. (1968) *J. Lipid Res.* **9**, 425.
[2] LEECH, R. M., RUMSBY, M. G. and THOMSON, W. W. (1973) *Plant Physiol.* **52**, 240.
[3] KANNANGARA, C. G., HENNINGSEN, K. W., STUMPF, P. K. and APPLEQVIST, L. A. (1972) *Plant Physiol.* **48**, 526.
[4] KIRK, J. T. O. and TILNEY-BASSETT, R. A. E. (1967) *The Plastids: Their Chemistry, Structure, Growth and Inheritance*, pp. 489–499, Freeman, London.

diversity.[5] The compositional and metabolic differences were much greater in relation to the morphological changes than those observed with tissue from dark-grown barley greened for varying lengths of time. The present work describes the characteristics of the chloroplastic fatty acid synthetase from maize leaves and examines fatty acid biosynthesis by plastids at successive stages of differentiation.

An outstanding problem of lipid biosynthesis in higher plants is the continued failure to obtain *de novo* synthesis of polyunsaturated fatty acids in isolated chloroplasts commensurate with the large amounts which occur endogenously. Possibly associated with this is the low incorporation of acetate into galactolipids since these are characteristically rich in linolenic and linoleic acids. In the present work attempts have been made to find conditions under which the *de novo* synthesis of unsaturated fatty acids by isolated chloroplasts is increased.

RESULTS

Conditions for optimum incorporation of acetate into lipids

Preliminary experiments showed that the incorporation of acetate into fatty acids by the relatively mature chloroplasts prepared from C, D and E sections of maize leaves was stimulated by the addition of ATP, CoA, bicarbonate and dithiothreitol (DTT) in amounts similar to those used by Stumpf and co-workers for spinach[6,7] and barley chloroplasts.[3] A co-factor requirement for Mg^{2+} could not be demonstrated because it was a component of the isolation medium.

Maximum rates of incorporation were obtained at short incubation times (Fig. 1) and the steady decline in rate with increased time may be attributable to a loss of chloroplast integrity. Incubations lasting for 30 min were used in subsequent experiments. In the absence of light there was an 8-fold decrease in the incorporation of acetate into lipid at 30 min.

There was a linear increase in acetate incorporation up to about 180 μg chlorophyll per tube: the incorporation then levelled off. In subsequent experiments the chlorophyll content of the chloroplasts added to each incubation mixture was kept below 180 μg and the acetate incorporation adjusted to a constant chlorophyll level (200 μg) in order to compare the effect of varying the experimental conditions.

At optimum ATP concentration (2 mM) acetate incorporation into lipids increased *ca* 2-fold to 7·3% compared with incubation mixtures in which there was no added ATP. Higher concentrations were inhibitory. Increasing the CoA concentration from zero to 0·6 mM gave a small stimulation (1·2 times), with a little difference in the range 0·2–0·6 mM. There was a sharp decline in the synthesis of fatty acids at pHs below 7·7 with an optimum in the region of pH 8·0 (Fig. 2). The ratios of unsaturated:saturated fatty acids synthesized were greatest at the pHs of optimum incorporation. Whereas synthesis of palmitate declined slightly when the pH was raised from 7·6 to 8·0, oleate synthesis increased from 370 to 540 pmol in 30 min. Similarly the synthesis of linoleate was enhanced by increasing the pH but even at the optimum pH only 60 pmol was synthesized from acetate.

Methods of isolation of chloroplasts

Lipid synthesis from acetate by maize chloroplasts prepared by the procedure outlined in the methods section compared favourably with the incorporation obtained by other

[5] HAWKE, J. C., RUMSBY, M. G. and LEECH, R. M. (1973) *Plant Physiol.* in press.
[6] KANNANGARA, C. G. and STUMPF, P. K. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1544.
[7] KANNANGARA, C. G. and STUMPF, P. K. (1972) *Arch. Biochem. Biophys.* **148**, 414.

workers using spinach chloroplasts (e.g. Stumpf and Boardman[8]). However, maize chloroplasts prepared using Honda's medium according to the method of Kannangara and Stumpf[7] for the preparation of active spinach chloroplasts were almost totally inactive (0·3% incorporation compared with 4·8% for spinach chloroplasts). The two techniques of isolation were equally effective in preparing active chloroplasts from spinach.

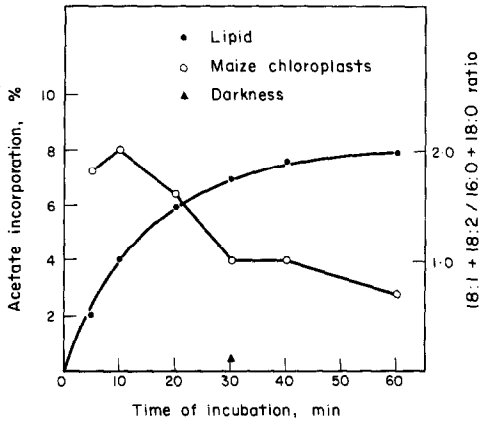

FIG. 1. THE EFFECT OF TIME OF INCUBATION ON THE INCOPORATION OF ACETATE-[1-^{14}C] INTO LIPID AND THE DISTRIBUTION OF RADIOACTIVITY IN THE UNSATURATED AND SATURATED FATTY ACIDS SYNTHESIZED BY ISOLATED MAIZE CHLOROPLASTS.
Incubation in darkness. See text for other conditions of incubation.

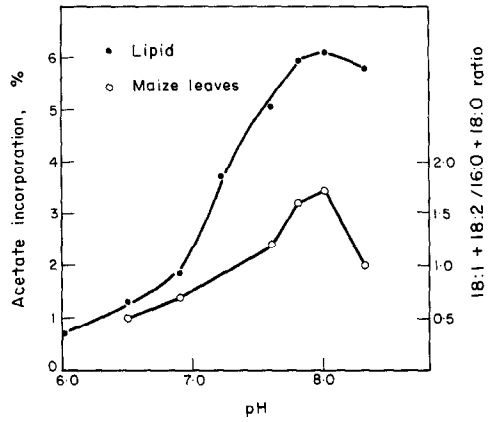

FIG. 2. EFFECT OF pH OF THE INCUBATION MEDIUM ON THE INCORPORATION OF ACETATE-[1-^{14}C] INTO LIPID AND THE DISTRIBUTION OF THE RADIOACTIVITY IN THE UNSATURATED AND SATURATED FATTY ACIDS SYNTHESIZED BY CHLOROPLASTS ISOLATED FROM MAIZE LEAVES.
See text for other conditions of incubation.

Effect of 3-(3,4-*dichlorophenyl*)-1,1-*dimethylurea* (*DCMU*), *Triton X*-100, *cyanide and arsenite*

DCMU(10 μM) slightly inhibited the incorporation of acetate into lipids by maize chloroplasts while 120 μM Triton X100 markedly inhibited incorporation (Table 1). The effect of Triton X100 examined in more detail showed that inhibition increased over the range 60–450 μM, while concentrations below 60 μM had little or no effect. With spinach chloroplasts 10 μM DCMU slightly stimulated and 120 μM Triton X100 slightly inhibited acetate incorporation. DCMU enhanced the synthesis of polyunsaturated fatty acids by spinach chloroplasts but in maize chloroplasts the distribution of label in the newly-synthesized fatty acids was not noticeably affected by this inhibitor. 1·0 mM cyanide and 1·0 mM arsenite slightly reduced total incorporation of acetate into lipid (Table 1). Separately these inhibitors had no marked effect on the distribution of label among the individual fatty acids but when added together there was an increased synthesis of linoleic acids and linolenic acids (9·8 and 8·4% respectively compared with normal levels of 3–4% for both fatty acids). However, there was an accompanying reduction in oleic acid synthesized so that the overall unsaturated fatty acid: saturated fatty acid ratio was uneffected.

Distribution of radioactivity in newly-synthesized fatty acids

A typical distribution of label in the newly-synthesized fatty acids of chloroplasts prepared from the total length of maize leaves and incubated with acetate-[1-^{14}C] under stan-

[8] STUMPF, P. K. and BOARDMAN, N. K. (1970) *J. Biol. Chem.* **245**, 2579.

dard conditions was as follows (mol%): 12:0(1·0), 14:0(3:2), 16:0 (40·4), 16:1 (1·9), 18:0(8·5), 18:1(41·1), 18:2(3·9), 18:3(trace). Despite the high proportions of endogenous linoleate and linolenate, very little of these polyunsaturated fatty acids was synthesized. Oleate and palmitate invariably predominated although the oleate:plamitate ratios were subject to variation in different preparations. Among several factors found to contribute to these variations were time of incubation, pH and temperature of the incubation medium. Unsaturated/saturated ratios were highest at short incubation times, falling from about 2·0 after 10 min to about 1·0 after 30 min (Fig. 1). Highest ratios were also obtained at pHs from 7·8 to 8·0 which coincided with the pH range of highest incorporation. Unsaturated/saturated ratios showed a 3-fold increase on raising the pH from 6·5 to 8·0 (Fig. 2). Slightly higher unsaturated/saturated ratios were obtained at 15° than at 20° (1·7 compared with 1·3).

TABLE 1. THE EFFECT OF DCMU, TRITON X100, ARSENITE AND CYANIDE ON ACETATE-[1-^{14}C] INCORPORATION INTO LIPIDS BY CHLOROPLASTS ISOLATED FROM SPINACH AND MAIZE LEAVES

Expt.	Chloroplasts	Incubation conditions	Incorporation (%)	Ratio: 18:1 + 18:2 + 18:3 / 16:0 + 18:0
1	Spinach	Standard	3·1	0·8
		+ DCMU	3·4	0·9
		+ Triton X100	2·8	0·9
		+ DCMU + Triton X100	5·9	1·9
2	Maize leaf (section D)	Standard	5·8	0·9
		+ DCMU	4·4	1·1
		+ Triton X100	0·6	1·2
		+ DCMU + Triton X100	0·5	0·8
3	Maize	Standard	8·5	1·4
		+ AsO_3'''	7·7	1·3
		+ KCN	7·7	0·9
		+ AsO_3''' + KCN	7·2	1·0

16·9 nmol (1 μCi)acetate, DCMU (10 μM), Triton X100 (120 μM), arsenite (1·0 mM) and cyanide (1·0 mM). See text for other conditions of incubation.

Incorporation of acetate into lipids by chloroplasts prepared from differentiating tissue

The estimated chlorophyll content of 150 × 10^6 plastids are 45, 50, 75, 125 and 200 μg chlorophyll respectively for plastids from A, B, C, D and E sections of maize leaves.[9] These chlorophyll values were used to compare the utilization of acetate for lipid biosynthesis by the same number of isolated plastids from each section.

There was a 5-fold increase in the incorporation of acetate into fatty acids by differentiating plastids from leaf section A through to leaf section D followed by a decreased incorporation of acetate by chloroplasts obtained from tissue of section E (Table 2). There were steady increases in synthesis of palmitate and palmitoleate up to section D but the extent of oleate and linoleate synthesis increased to a greater extent to give a linear rate of increase from sections A–D and a 12-fold increase. Despite the large proportions of linolenate as a constituent of endogenous lipids of plastids isolated from the different sections

[9] LEESE, B. M., LEECH, R. M. and THOMSON, W. W. (1971) *Proc. 2nd Int. Congress on Photosynthesis*, Vol. II, pp. 1486–1494. Stresa, Ed. JUNK, The Hague.

(Table 3) polyunsaturated biosynthesis was always low in the numerous experiments carried out. Although linolenate was below the level of detection in the experiment cited in Table 2, up to about 8% linolenate was obtained in other experiments (see Table 5).

TABLE 2. THE INCORPORATION OF ACETATE-[1-^{14}C] INTO FATTY ACIDS BY PLASTIDS PREPARED FROM LEAF SECTIONS OF 7-DAY-OLD MAIZE SEEDLINGS

Leaf section	Incorporation of acetate into lipid/ 150×10^6 plastids (%)	(pmol)	Incorporation of acetate into fatty acids (pmol) 12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	Ratio: Unsaturated / Saturated
A	1·4	250	5	19	151	10	—	65	—	—	0·4
B	2·8	480	—	8	255	20	—	184	13	—	0·8
C	4·4	740	3	15	326	24	—	350	22	—	1·2
D	7·7	1300	—	17	321	56	55	781	70	—	1·4
E	3·0	510	6	14	218	27	31	192	22	—	0·9

(—) None detected.

Details of reaction mixture given in text. 16·9 nmol acetate-[1-^{14}C](1 μCi) was used and each suspension contained *ca.* 150×10^6 plastids. Incubations were for 30 min at 27 000 lx at 20°.

Distribution of radioactivity in lipids

The fatty acids synthesized from acetate-[1-^{14}C] by chloroplasts were mainly present in three lipid fractions, namely free fatty acids, polar lipids and a component which chromatographed with 1·2-diglyceride(DG) in three eluting solvents on TLC. PC was the main radioactive constituent of the polar lipids and only small amounts of mono- and di-galactosyldiglyceride were synthesized. The newly-synthesized free fatty acids were much more unsaturated than the fatty acids of the other two major radioactive components (Table 4). Most of the radioactivity in DG occurred as palmitate and in the polar lipids 45·6 and 32·0% of the ^{14}C-label was in palmitate and oleate respectively.

TABLE 3. DISTRIBUTION OF THE TOTAL FATTY ACID IN PLASTIDS PREPARED FROM SECTIONS A–E OF MAIZE LEAVES

Leaf section	% distribution of FA s 16:0	18:0	18:1	18:2	18:3
A	28·3	12·1	14·3	19·1	26·2
B	11·3	2·2	8·7	9·8	68·0
C	9·8	3·8	3·4	4·4	78·6
D	16·2	5·2	7·4	4·0	67·2
E	19·6	1·7	3·8	5·0	69·9

Effect of non-chloroplastic particulate fractions on synthesis of fatty acids by chloroplasts

The addition of the non-chloroplastic particulate fraction of maize leaves to the standard incubation mixtures stimulated the incorporation of acetate into fatty acids by chloroplasts and increased the synthesis of unsaturated fatty acids (Table 5). Little or no synthesis of fatty acids occurred when chloroplasts were omitted. The extent of enhancement varied from being quite small to more than a doubling of the total synthesis. Perhaps more significant were the 4-fold increases in oleate synthesis which frequently occurred and the minor increases in linoleate and linolenate. This stimulatory effect was greatest when tissue

TABLE 4. THE DISTRIBUTION OF LABEL IN THE FATTY ACIDS OF THE MAIN CLASSES OF LIPID SYNTHESIZED BY MAIZE CHLOROPLASTS FROM ACETATE-[1-^{14}C]

Lipid class	Distribution of total radioactivity (%)	% of total activity in FAs							
		12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3
Free fatty acid	53·6	—	0·7	9·4	2·0	4·7	76·4	4·6	2·2
Diglyceride	27·9	—	1·8	86·5	2·5	2·2	5·6	1·4	—
Polar lipid	18·5	5·0	7·4	45·6	1·7	5·9	32·0	2·4	—

(—) None detected.

Chloroplasts prepared from all the leaf tissue of 7-day-old plants. Details of reaction mixture and incubation conditions given in text.

TABLE 5. THE EFFECT OF NON-CHLOROPLASTIC PARTICULATE FRACTION (100 000 *g* PELLET) ON SYNTHESIS OF LONG CHAIN FATTY ACIDS FROM ACETATE-[1-^{14}C] BY MAIZE CHLOROPLASTS

Section of leaf	Total incorporation (pmol)*		Incorporation into fatty acids† (pmol)					Ratio: Unsaturated / Saturated
	Chloroplasts alone	Chloroplasts + particulate fraction	16:0	18:0	18:1	18:2	18:3	
A–E	1170		437	122	519	25		0·97
		1370	354	93	750	60		1·81
A–D	1190		379	77	616			1·35
		1120	230	23	741	37		3·08
A–D	760		176	33	513	40		2·65
		870	167	39	559	38		2·90
D	1300		321	55	781	70		2·26
		1400	381	22	882	55		2·33
E	490		209	30	184	22		0·86
		750	251	25	391	42		1·57
D	1220		338		694	95		2·33
		850	180	14	553	62		3·17
C–D	440		209	33	117	19	31	0·69
		510	118	17	240	43	43	2·41
E	510		197	41	181	19	32	0·97
		980	184	40	654	40	32	3·24
C–E	660		259	97	226	15	24	0·74
	680		223	95	288	25	16	1·03
		1810	337	156	1059	78	85	2·52
		1600	336	82	997	83	48	2·70
C–E	470		221	61	139	15		0·55
		900	201	41	564	46	30	2·64
C–E	1040		422	200	339	21		0·58
		1230	340	140	673	22	6	1·46

Details of reaction mixture and incubation conditions given in text. 16·9 nmol acetate-[1-^{14}C] (1 μCi) was used.

* Non-chloroplastic particulate fraction alone incorporated about 30 pmol.

† Small synthesis of 12:0, 14:0 and 16:1 not included.

from section E was used to prepare the chloroplasts, either alone or with tissue from other sections. The synthesis of saturated fatty acids was either unaffected or slightly reduced by the addition of the particulate fraction so the accompanying stimulation of biosynthesis of unsaturated fatty acids invariably resulted in greater unsaturated:saturated ratios.

As shown in Fig. 3a, the magnitude of the stimulation was dependent on the amount of particulate fraction added, where 0·5 ml of particulate fraction was equivalent to about the same cellular levels relative to the amount of chloroplasts used in the experiments. Beyond this level the relative increases in fatty acid biosynthesis were only small. Similar overall effects on fatty acid synthesis by chloroplasts were obtained by mitochondrial and microsomal subfractions: both increased the synthesis of oleate to a similar extent (Fig. 3b). NADPH and NADH, either together or separately, stimulated the biosynthesis of oleate by chloroplasts alone and by chloroplasts in the presence of non-chloroplastic particulate fractions (Table 6). The incorporation of acetate into fatty acids by non-chloroplastic fractions alone was negligible in every experiment. These fractions were pigmented and contained chloroplast fragments. No intact chloroplasts were observed under phase-contrast microscopy.

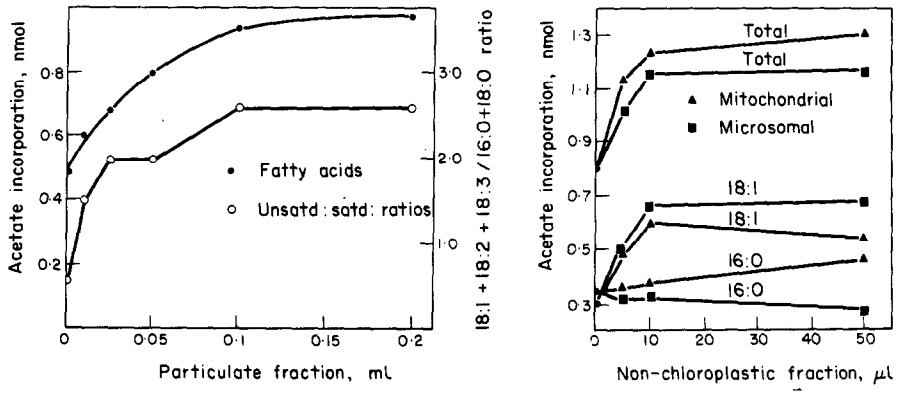

FIG. 3. EFFECT OF NON-CHLOROPLASTIC CELL FRACTIONS ON THE SYNTHESIS OF FATTY ACIDS FROM ACETATE-[1-^{14}C] BY CHLOROPLASTS ISOLATED FROM MAIZE LEAVES.
(a) Total particulate fraction: synthesis of total fatty acids and unsaturated:saturated ratios. (b) Mitochondrial and microsomal fractions. See text for other conditions of incubation.

Very few contaminating mitochondria were observed in the chloroplasts prepared by the standard method outlined above. The inclusion of an additional gradient centrifugation during the preparation of the chloroplasts did not alter the palmitate/oleate ratio. Omission of the 0·6 M sucrose gradient gave slightly higher incorporation of acetate into fatty acids (7·0% compared with 6·1%) and gave a higher oleate/palmitate ratio (1·03 compared with 0·82). These effects may be attributable to a contamination of chloroplasts by mitochondrial and microsomal particles when a gradient centrifugation was omitted. The addition of particulate fractions did not noticeably alter the distribution of the label in the radioactive products, i.e. free fatty acids, polar lipid and DG. The greatest stimulation of oleic acid biosynthesis occurred in the free fatty acid fraction.

Effect of metabolites and supernatant fraction

Although it is clear from the above results that chloroplasts are the site of *de novo* synthesis of fatty acids, the stimulation of desaturation caused by other cell organelles presup-

poses the transfer of fatty acids, or appropriate derivatives, within the cell. The addition of soluble cell components to the standard incubation mixtures using chloroplasts alone slightly reduced the total incorporation of acetate into long chain fatty acids but did not alter the proportions of the unsaturated and saturated fatty acids synthesized (Table 7). When supernatant and mitochondrial fractions were both added there was a slight reduction in the total incorporation of acetate into long chain fatty acids and a marked reduction in the proportions of unsaturated fatty acids synthesized, but the total synthesis of the latter remained considerably greater than with chloroplasts alone.

TABLE 6. THE EFFECT OF NADH AND NADPH AND MITOCHONDRIA ON ACETATE-[1-^{14}C] INCORPORATION BY CHLOROPLASTS ISOLATED FROM MAIZE LEAVES

Source of chloroplasts	Condition	Incorporation of radioactivity into total lipids (pmol)	Radioactivity in FAs (pmol)					Unsaturated / saturated
			16:0	18:0	18:1	18:2	18:3	
D section	Standard minus NADH and NADPH	1220	338	–	694	95	–	2·3
	NADH	1380	312	72	677	143	–	2·1
	NADPH	930	219	20	618	28	–	2·7
	Mitochondria	850	180	14	553	62	–	3·2
	NADH and mitochondria	990	174	17	729	33	–	4·0
	NADPH and mitochondria	870	133	15	634	45	–	4·3
C, D and E sections	Standard minus NADH and NADPH	910	358	106	350	38	23	0·9
	Mitochondria	1300	465	48	541	33	30	1·2
	Mitochondria, NADH and NADPH	989	253	8	566	35	16	2·4
C, D and E sections	Standard minus NADH and NADPH	470	221	61	139	15	–	0·5
	NADH and NADPH	400	142	49	187	7	8	1·1
	Mitochondria	790	222	31	360	45	27	1·7
	Mitochondria, NADH and NADPH	750	155	32	494	36	16	2·9

(–) None detected.

0·2 mM NADH and NADPH was omitted from the standard incubation mixture where indicated. 16·9 nmol acetate-[1-^{14}C] (1 μCi) was used. The relative amounts of chloroplast and mitochondria were approximately in proportion to the amounts found in cells. See text for incubation conditions.

TABLE 7. EFFECT OF ADDITION OF SUPERNATANT CELL FRACTION, CARNITINE AND ACETYL CoA: ACETYL CARNITINE TRANS-FERASE ON THE INCORPORATION OF ACETATE-[1-^{14}C] INTO FATTY ACIDS BY CHLOROPLASTS

Experiment	Addition to standard conditions of incubation	Incorporation of radioactivity (pmol)							Unsaturated / saturated
		Total lipids	14:0	16:0	18:0	18:1	18:2	18:3	
1	Nil	760	14	176	33	513	40	40	2·7
	Carnitine	891	11	296	43	474	37	–	1·5
	Carnitine and transferase	270	12	164	10	69	–	–	0·4
	Mitochondria	870	4	167	39	559	38	46	3·1
	Mitochondria and carnitine	828	11	208	39	504	–	–	2·0
	Mitochondria, carnitine and transferase	73	4	37	3	24	–	–	0·5
2	Nil	1500	48	606	128	617	59	–	0·9
	Transferase	473	49	266	28	88	–	–	0·3
	Carnitine and transferase	406	19	174	48	112	19	–	0·5
	Supernatant	1031	19	316	167	444	37	22	1·0
	Mitochondria	2129	34	453	79	1171	194	128	2·1
	Mitochondria and carnitine	2096	42	530	75	1157	136	92	2·6
3	Nil	670	–	242	96	257	20	20	0·9
	Mitochondria and microsomes	1710	–	338	119	1028	90	47	2·6
	Mitochondria, microsomes and supernatant	1627	–	462	171	900	52	20	1·5

(–) None detected.

See text for standard conditions of incubation. 16·9 nmol acetate-[1-^{14}C] was used. Leaf sections used in expts. 1, 2 and 3 were A–D, A–E and C–E respectively.

Carnitine addition was without effect and acetyl CoA: acetyl carnitine transferase markedly inhibited incorporation of acetate into lipid and in particular considerably reduced the synthesis of unsaturated fatty acids (Table 7). This non-specific inhibition occurred both in the presence and absence of mitochondria and carnitine. Supplementing the incubation mixtures with glycerophosphate and citric acid cycle intermediates altered neither the total incorporation nor the ratios of fatty acids synthesized.

DISCUSSION

In the past, difficulties have been experienced in obtaining appreciable lipid synthesis from acetate by chloroplasts from Gramineae species,[3,10] but in the present experiments the activity of the synthetase of maize chloroplasts is comparable with that of spinach chloroplasts.[7,10] The isolation procedure appears to be highly important in the retention of activity and the procedure using Honda's solution,[8] although suitable for spinach chloroplasts, gave inactive maize chloroplasts. The special features of the isolation procedure which contributed to the preparation of a high proportion (60%) of Class I chloroplasts,[9] the organelles with the highest capacity for fatty acid synthesis,[8] was the inclusion of bovine serum albumin in the isolation medium, brief homogenization followed by rapid filtration and rapid removal of the supernatant by short-term centrifugation. However, the co-factor requirements for the fatty acid synthetase in maize chloroplasts, like the synthetase in other higher plants,[7] include ATP, CoA, bicarbonate and a disulphide reducing agent such as DTT.

The detailed description of the ultrastructural differences in plastid structure of the developing maize leaf[2] makes it possible to relate the capabilities of chloroplasts for lipid biosynthesis to a particular phase of differentiation. Section C marks the transition between the proplastid-containing tissue (sections A and B) and the tissue in sections D and E containing mesophyll chloroplasts (grana of 12–15 compartments) and agranal bundle sheath chloroplasts.[2] Parallel with these morphological changes, the acetate utilization for total lipid synthesis by an equivalent number of plastids increased with increasing differentiation (sections A–D) and decreased on attainment of chloroplast maturity (section E). Kannangara *et al.*[3] observed a similar but much less pronounced trend in 7-day-old barley grown in the dark, namely that acetate incorporation increased with increasing time of greening up to a maximum of 12 hr and then decreased. Chloroplasts prepared from immature spinach leaves also had a greater capacity for lipid synthesis than chloroplasts prepared from mature leaf tissue[7] but the two types of chloroplast were compared at a constant chlorophyll level and it is likely that the greater activity of the chloroplasts from immature tissue is attributable, at least in part, to the presence of a greater number of plastids in the incubation mixtures.

Numerous studies have shown that appreciable oleate synthesis occurs in Class 1 chloroplasts but attempts to obtain conditions which produce extensive synthesis of linoleate and linolenate, the principal fatty acids in chloroplasts, have been unsuccessful. The highest desaturase activity in isolated maize chloroplasts appears to be associated with the later phases of chloroplast differentiation and granal formation (sections C and D in the present experiments). Although the growth characteristics of spinach leaves do not allow the selection of clearly defined regions of development, it is relevant that Kannangara and Stumpf[6,7] obtained improved synthesis of linoleate and linolenate by using young rather

[10] KANNANGARA, C. G. and STUMPF, P. K. (1972) *Plant Physiol.* **49**, 497.

than mature spinach leaves as a source of chloroplasts. Recently Stumpf *et al.*,[11] using disrupted spinach chloroplasts, have provided evidence for the synthesis of linolenate by addition of acetate to *cis* 7,10,13-hexadacatrienoate. Linoleate was not synthesized so that C_{18} dienoic and trienoic acids appear to be synthesized by different pathways.

However, both with spinach and maize, the linoleate and linolenate content of the fatty acids synthesized by intact chloroplasts from acetate falls far short of the proportions which occur endogenously although ATP, CoA and Triton X100,[6,7] enhanced the synthesis of unsaturated fatty acids, principally oleate, by spinach chloroplasts. ATP and CoA stimulated total synthesis of fatty acids by maize chloroplasts but there was no noticeable alteration in the distribution of label in the newly-synthesized fatty acids. If chloroplasts alone are involved in these syntheses, the stimulated rate of fatty acid formation suggests that under these conditions the transport of ATP and NAD(P)H across the chloroplast envelope is of the order of 1 nmol/hr. Triton X100 depressed fatty acid synthesis without altering the distribution of label. As found earlier with spinach chloroplasts[8,12] addition of DCMU, an inhibitor of noncyclic electron transport, slightly inhibited fatty acid synthesis by maize chloroplasts and increased desaturation but cyanide did not inhibit linoleate synthesis as with spinach chloroplasts.[7] Decreased total fatty acid synthesis at low pHs resulting from impaired oleate synthesis suggests lability of the enzymes catalyzing the transformation of palmitate to oleate, as does the relatively greater monoenoic synthesis at short incubation times.

The desaturation of oleate by chloroplasts isolated from higher plants has not been demonstrated directly although an impure chloroplast fraction of *Chlorella vulgaris* converted oleoyl CoA to linoleate by a reaction which requires NADPH and O_2 and appeared to involve PC as an intermediate.[13,14] Further indirect evidence for the involvement of PC is provided by the high specific activity and turnover of this lipid in pumpkin leaves which utilized acetate-[1-^{14}C] for synthesis of linoleic.[15] However, in non-chloroplastic tissues from plants[16] and in animal tissues[17] desaturases are microsomal and Vijay and Stumpf[18] have demonstrated that oleoyl CoA and not phospholipid is the specific substrate for the microsomal desaturase of safflower seeds. Subsequent transfer of the desaturated oleoyl group to PC appears to be catalysed by a very active microsomal transacylase.[18] Abdelkader and Mazliak[19] have shown that phospholipid exchange between mitochondria and microsomes of potato and cauliflower is mediated by the cytoplasmic supernatant and it is possible that similar exchanges occur in photosynthetic tissues between chloroplasts, mitochondria and microsomes to achieve the synthesis of the full complement of tissue lipids from precursors such as acetate.

Inclusion of a gradient centrifugation, following the initial centrifugation, to precipitate chloroplasts gave a chloroplastic preparation with low contamination by other particulate material of the cell so that the interaction of organelles of leaf tissue in achieving fatty acid and lipid synthesis could be examined. Consistent enhancement of fatty acid synthesis and incorporation of label into unsaturated fatty acids, principally oleate and to a lesser extent

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[18] VIJAY I. K. and STUMPF P. K. (1971) *J. Biol. Chem.* **246**, 2910.
[19] BEN ABDELKADER, A. and MAZLIAK, P. (1970) *European J. Biochem.* **15**, 250.

linoleate and linolenate, was obtained by adding non-chloroplastic particulate material: NADH and NADPH further stimulated synthesis of unsaturated fatty acids. Both mitochondrial and microsomal-rich fractions produced the enhancement effect which emphasizes the need to work with carefully purified chloroplasts when fatty acid synthesis by this organelle alone is being investigated.

EXPERIMENTAL

Plant material. Zea mays (var. Kelvedon Glory) was grown for 7 days in constant environment cabinets, at 28° and 25 000 lx ($0{\cdot}8 \times 10^{-5}$ erg/sec/cm^2) as described elsewhere.[5] After removal of the coleoptile and first leaf, the remaining leaves (usually 10–14 cm long) were cut transversely into 5 sections—the four lower sections (A–D) each of 2 cm and the remaining section (E) about 4 cm. The distal tissue of leaves more than 12 cm long was discarded.

Spinach (*Spinacia oleracea* L.) was grown in controlled environment cabinets at 22° with a 5° night depression at 25 000 lx ($0{\cdot}8 \times 10^{-5}$ erg/sec/cm^2) and a 12 hr light period.

Isolation of chloroplasts. Routinely the method developed by Leese *et al.*[9] to give a high proportion of chloroplasts having intact double outer envelopes with little or no contamination by non-chloroplastic material was used to prepare chloroplasts from the leaf sections of maize. The procedures with tissue from leaf sections A and B and tissue from leaf sections C, D and E were slightly different. Leaf sections C, D and E were blended in an ice-cold medium consisting of 0·5 M sucrose in 0·067 M phosphate buffer at pH 8·0 containing 1 mM $MgCl_2$ and 0·2% bovine serum albumin (Cohn Fraction V) using a MSE Atomix at full speed for 3 sec and then 5 sec with remixing between blends. The homogenate was filtered through 10 layers of cotton organdie and 10 layers of 25 μ nylon bolting cloth and the filtrate immediately centrifuged for 1 min at 3000 g. The pellet was resuspended in isolation medium, filtered through one layer of Miracloth (Calbiochem) and 5 ml aliquots layered onto 20 ml 0·6 M sucrose in 50 ml centrifuge tubes in the buffer described above. Centrifugation was carried out at 2000 g for 15 min. The chloroplast from the latter centrifugation was added to incubation mixtures as a suspension in 0·6 M sucrose-buffer. Tissue from A and B leaf sections was ground in ice-cold 0·5 M sucrose-buffer with a pestle and mortar, and the brei filtered through 5 extra layers of 25 μ nylon bolting cloth before centrifugation of the filtrate at 440 g for 7 min to remove cell debris. The supernatant was then centrifuged for 90 sec at 3000 g before continuing with the gradient procedure described above for the preparation of chloroplasts from sections C–E. The method described by Kannangara and Stumpf[6] using Honda's medium was used in a few preparations of maize and spinach chloroplasts. Chlorophyll was determined by the method of Arnon.[20]

Preparation of non-chloroplastic particulate fractions and cell supernatant. The supernatant obtained by centrifugation at 3000 g of the homogenate from leaf sections C–E during the preparation of chloroplasts, was centrifuged at 4000 g for 10 min and the supernatant centrifuged at 20 000 g for 30 min to precipitate the mitochondrial fraction. The resulting supernatant was then further centrifuged at 100 000 g for 60 min to prepare a microsomal fraction. Alternatively, a total non-chloroplastic particulate pellet was prepared from the 4000 g supernatant by centrifugation at 100 000 g for 60 min. All pellets were resuspended in the same volume of 0·5 M sucrose isolation buffer at pH 8·0 as the corresponding preparations of chloroplasts. The cell supernatant was obtained by homogenizing maize leaves in four volumes of 0·067 M phosphate buffer at pH 8·0 and centrifuging at 100 000 g for 30 min. Freeze-dried preparations were resuspended in *ca.* 5 times the vol. of 0·1 M tricinephosphate buffer at pH 7·8. used in the reaction mixture.

Chemicals. Na acetate-[1-^{14}C] (59 mCi/mmol) was purchased from Amersham Radiochemical Centre (Bucks). DCMU was a gift from ICI Ltd.

Reaction mixtures. Chloroplasts (100–180 μg chlorophyll) suspended in 0·1 ml 0·5 M sucrose, isolation buffer were incubated in the following reaction mixture except where otherwise indicated: 300 mM sorbitol, 50 mM tricine at pH 7·8, 50 mM potassium phosphate at pH 7·8, 2·5 mM DTT, 2 mM ATP, 30 mM $NaHCO_3$, 0·5 mM CoA, 0·5 mM $MgCl_2$, 0·2 mM NADH, 0·2 mM NADPH and 16·9 nmol Na acetate-[1-^{14}C] (1 μCi), to a total volume of 1·0 ml in 10 ml ground glass tubes with round bases. The tubes were attached to a circulating disc inclined at about 20° from the vertical axis during 30 min incubations in a glass water-bath. The light intensity at the tube surface was 27 000 lx (ten-150 W Osram Filtra-lite lamps) at 20°. The reaction was stopped by the addition of 0·1 ml 5 M HCl and 5 ml $CHCl_3$–MeOH (2:1).

Isolation and analysis of lipids. Extraction and purification of the lipids was carried out in the reaction tubes by washing the $CHCl_3$ layer in turn with 1% by vol. HOAc, 0·1 M NaCl and 3× with H_2O. The final $CHCl_3$ solution was dried under a stream of N_2 at room temp. and the residue redissolved in a suitable vol. of $CHCl_3$ for subsequent analysis. Methods used for the measurement of radioactivity, TLC of reaction products and GLC of methyl esters have been described.[5]

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